Bioavailable vitamin D levels are reduced and correlate with bone mineral density and markers of mineral metabolism in adults with nephrotic syndrome

Abhinav Aggarwal^{1*}, Ashok K Yadav^{2*}, Raja Ramachandran^{2*}, Vinod Kumar², Vivek Kumar², Naresh Sachdeva³, Niranjana Khandelwal⁴, Vivekanand Jha^{2, 5, 6}

Departments of ¹Internal Medicine, ²Nephrology, ³Endocrinology, and ⁴Radiodiagnosis, Postgraduate Institute of Medical Education and Research, Chandigarh, India, ⁵George Institute for Global Health, New Delhi, India and ⁶University of Oxford, Oxford, UK.

**Equal contributions*

Running Head:

Bioavailable vitamin D and BMD in nephrotic syndrome

Correspondence to: Prof Vivekanand Jha, George Institute for Global Health, 219-221 Splendor Forum, Jasola District Center, New Delhi 110 025, INDIA Email: vjha@pginephro.org

Abstract

Background: Blood levels of 25-hydroxyvitamin D [25(OH) D] are reduced in patients with nephrotic syndrome (NS). The lowering is thought to be due to urinary loss of vitamin D binding protein (DBP). A link between vitamin D deficiency and bone disease or markers of mineral metabolism has not yet been shown in NS. We hypothesized that alterations in bioavailable vitamin D levels might be linked to these abnormalities in NS.

Methods: We measured circulating levels of 25(OH)D, 1,25-dihydroxyvitamin D [1,25(OH)₂D], DBP, serum albumin and intact parathyroid hormone (iPTH) in 106 adults with sporadic idiopathic NS and 40 healthy controls. Bioavailable vitamin D were calculated from previously validated formulae. Bone mineral density (BMD) was measured at left hip (neck of femur) by DEXA.

Results: Compared to healthy controls, total and bioavailable 25(OH)D levels were significantly reduced in patients with NS as compared to healthy controls. Among the nephrotic patients, BMD was positively correlated with bioavailable 25(OH)D ($r = 0.358$; $p = 0.0002$) but not with total 25(OH)D ($r = 0.174$; $p = 0.079$). Total 1,25(OH)₂D and bioavailable 1,25(OH)₂D did not correlate with BMD ($r = 0.131$; $p = 0.206$ and $r = 0.107$, $p = 0.295$). Bioavailable 25(OH)D levels showed a strong inverse correlation with iPTH on univariate ($r = -0.457$; $p < 0.0001$) and multivariate ($\beta = -0.453$, $p < 0.0001$) analyses.

Conclusions: We conclude that bioavailable 25(OH)D is a better measure of vitamin D status with respect of BMD and mineral metabolism in patients of nephrotic syndrome.

Keywords: Nephrotic syndrome, vitamin D, bone mineral density, parathyroid hormone, bioavailable

Introduction

Patients with nephrotic syndrome (NS) are reported to have low concentrations of 25 (OH) vitamin D, the major circulating form of vitamin D^{1, 2}. The depletion of vitamin D binding protein in the urine is thought to lead to this state of vitamin D deficiency³. Despite the low serum concentrations of 25 (OH) vitamin D, data on the prevalence of bone disease and/or hyperparathyroidism in these patients are conflicting⁴⁻⁷.

The concept of free hormone hypothesis, well-established for steroid hormones like testosterone, has been proposed for this pleotropic hormone. According to this hypothesis, it is the free rather than total concentration of vitamin D that truly reflects the biologically active fraction of the hormone⁸. The major portion of circulating 25(OH)D and 1,25(OH)₂D is tightly bound to DBP, a very small fraction is bound to albumin, and less than 1% of circulates in free, unbound form⁸⁻¹⁰. The binding to albumin is loose, hence this fraction is also available for action at cellular level¹¹. It has therefore been suggested that the bioavailable (free + albumin-bound) level might be a more accurate index of vitamin D sufficiency^{11, 12}. A closer association has been shown between the bioavailable vitamin D levels and bone mineral density in healthy individuals and indices of mineral metabolism in hemodialysis patients^{11, 13}.

The validity of the free hormone hypothesis with regard to vitamin D has not yet been examined in patients of nephrotic syndrome. The primary abnormality in NS is a loss of circulating albumin in urine, leading to varying degrees of hypoalbuminemia. Since albumin circulates in much larger concentrations compared to DBP, loss of this molecule is likely to have a greater effect on the levels of bioavailable vitamin D. We hypothesized that variations in vitamin D levels in NS could be mediated by alterations in circulating levels of albumin and impact the relationship between vitamin D status and mineral metabolism.

Material and Methods

This cross-sectional study was carried out from July 2011 to December 2014 at the Nehru Hospital, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Adult patients (age >18 yrs) with newly diagnosed NS (defined as proteinuria >3.5 g/d or >1.5 g/d along with serum albumin <2.5 g/dl, edema, and hyperlipidemia) were included¹⁴. Those with secondary NS, serum creatinine >1.4 mg/dl, pregnant or lactating females, evidence of chronic diseases like diabetes mellitus, heart failure, liver disease, recent infections including hepatitis B, hepatitis C and HIV- I/II, any other cause of proteinuria and history of recent or long term intake of steroids, cytotoxic agents, antiepileptics, calcium, vitamin D supplements bisphosphonates, steroids or immunosuppressive therapy were excluded. Patients who fulfilled all inclusion and exclusion criteria were enrolled in the study. We also recruited a total of 40 healthy controls from general population. All controls were over 18 years old with normal renal function and no co-morbidities. The study was approved by Institute Ethics Committee, and all the participants provided a written informed consent.

Participants were evaluated using a standardized clinical interview, physical examination and laboratory tests. Ten ml venous blood sample was withdrawn and the serum stored at - 80°C for analysis. Kidney biopsy was done to establish the diagnosis in all except 2 cases, who were presumed to have MCD on clinical grounds.

Biochemical assays

Total 25 (OH) D was measured in duplicate using a commercially available EIA kit. (IDS Laboratory, UK). The sensitivity of this assay is 5 nmol/L. The intra and interassay coefficients of variation of this assay were 5.3 - 6.7% and 4.6 - 8.7% respectively. Accuracy of this assay

is comparable with recognised radioimmunoassay for 25 (OH) D ($r=0.9$). Vitamin D status was defined as deficient (< 25 nmol/L), insufficient (25-75 nmol/L) and normal or sufficient (> 75 nmol/L). Total 1,25(OH)₂D was measured by commercially available EIA kit (IDS Laboratory, UK). The sensitivity of this assay is 2.5 pmol/L. The intra and interassay coefficients of variation were 9.3 - 10.7% and 17.6 - 19.1% respectively. Assay accuracy is equivalent to 1, 25 (OH)₂ D radioimmunoassay ($r=0.95$).

DBP was measured in duplicate in serum and urine samples by commercial ELISA kit (R&D Systems, Minneapolis, MN, catalog number DVDBP0) according to the manufacturer's instructions. The assay was conducted after diluting serum samples to 1 to 2000 in Calibrator Diluent RD6-11 (R&D Systems, # 895489). The intra and inter-assay coefficients of variation were 6.2% at 180 ng/mL and 6% at 105 ng/mL respectively. The sensitivity of the assay was 3.74 ng/mL. The manufacturer reported no significant cross-reactivity with human albumin or vitamin D₃. Urinary DBP was corrected for creatinine and results expressed as DBP/creatinine ratio .

Intact PTH was measured using Roche Elecsys PTH Test System analyzer. The intra and inter-assay coefficients of variation were 5.4% and 5.9% respectively.

Serum calcium, phosphorus and albumin were measured on the Cobas C111 automated analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Bioavailable 25 (OH) D was determined using modified Vermeulen equations, which have been validated for calculation of unbound testosterone. This method defines free, bioavailable (free + albumin bound) fraction and DBP bound fraction ^{11, 15}. Vitamin D Binding affinity

constants was used as estimated by equilibrium dialysis and centrifugal ultrafiltration dialysis ($7 \times 10^8 \text{ M}^{-1}$ for DBP and $6 \times 10^5 \text{ M}^{-1}$ for albumin) ⁹. Bioavailable 1,25(OH)₂D was also calculated using same approach. In this study we used the 1,25(OH)₂D binding affinity constants previously derived by centrifugal ultrafiltration dialysis ($3.7 \times 10^7 \text{ M}^{-1}$ for DBP and $5.4 \times 10^4 \text{ M}^{-1}$ for albumin) ¹⁰.

Bone mineral density

All subjects underwent dual-energy X-ray absorptiometry (DXA; Discovery Wi, Hologic, Waltham, MA, USA) to determine regional BMD. Hologic phantoms were used to calibrate the instrument. We used left hip in this study as the measure of BMD. The results of the scan gave a T – score and bone density in terms of gram/cm². The interpretation of the T - score was done as per the WHO classification ¹⁶. The inter-subject variability was $\pm 1\%$.

Statistical analysis

Data are presented as mean $\pm$ SD and range. Data were tested for normality of distribution. Continuous variables were compared with independent samples Student's t test if normally distributed, else with Mann-Whitney U test. Correlations were tested with Pearson and Spearman correlation tests as appropriate. Two-tailed p-value <0.05 were considered as significant. Comparisons were made between control and study population. Correlations between bioavailable and total vitamin D, DBP, degree of proteinuria and hypoalbuminemia, eGFR, calcium, phosphate, parathyroid hormone and BMD were investigated. Multiple regression analysis was performed to predict independent factors. All analyses were conducted using the SPSS software version 16.0 (IBM).

RESULTS

Demographic and clinical characteristics of study subjects

The demographic and clinical characteristics of the study subjects are shown in Tables 1 and 2. There were no differences in age, gender and body mass index (BMI) of the patient and control groups. The majority (65%) of the patients with NS were males, and the mean age at onset of NS was 37.89 ± 13.54 years. The median duration of symptoms before presentation was 3 months. Of the 106 patients who underwent a biopsy, 48 (45.3%) had membranous glomerulonephritis, 20 (18.9%) had focal segmental glomerulosclerosis, 10 (9.4%) had minimal change disease (MCD), 6 (5.6%) had IgA nephropathy and 4 (3.7%) had C1q nephropathy (C1qN). Other conditions included C₃ glomerulonephritis, lupus nephritis, amyloidosis, fibrillary glomerulonephritis, collapsing glomerulonephritis and mesangiocapillary glomerulonephritis. Two patients who did not undergo biopsy were presumed to have MCD on clinical grounds. There was no difference in serum creatinine and eGFR values of the patient and control groups. The eGFR was <60 ml/min in 5 patients with NS.

The mean BMD was 0.79 ± 0.15 g/cm² and 0.80 ± 0.11 g/cm² in NS and controls respectively ($p=0.78$). A total of 40 (37.7%) patients had osteopenia and 2 (1.9%) had osteoporosis in the NS group and 12 (30%) had osteopenia and 1 (2.5%) had osteoporosis amongst controls.

Vitamin D levels

Table 3 shows the 25(OH)D levels in the two groups. A total of 74% NS subjects were 25(OH)D deficient and 26% were insufficient. In comparison, 37.5% HC were 25(OH)D deficient and 57.5% were insufficient. There was wide variation in levels of DBP and total 25(OH)D in both groups.

Patients with NS had lower total 25(OH)D levels compared to controls (21.39 ± 8.99 versus 29.64 ± 11.61 nmol/L; $p<0.0001$). Importantly, the bioavailable 25(OH)D levels were lower in

patients with NS compared to controls (1.59 ± 1.22 versus 4.93 ± 4.15 nmol/L; $p < 0.0001$). The level of $1,25(\text{OH})_2\text{D}$ was also lower in NS subjects compared to HC (52.39 ± 66.53 vs 114.12 ± 111.00 pmol/L, $p < 0.0001$). Similarly, the bioavailable $1,25(\text{OH})_2\text{D}$ was lower in NS as compared to healthy subjects (9.12 ± 15.47 vs 23.08 ± 18.32 pmol/L, $p < 0.0001$).

Body mass index (BMI) was not associated with any measure of vitamin D both in NS and control subjects. Although NS patients with $\text{BMI} \geq 25 \text{ kg/m}^2$ ($n=33$) had lower levels of all forms of vitamin D and higher DBP levels, this difference did not reach statistical significance (data not shown).

The ratio of bioavailable to free $25(\text{OH})\text{D}$ was 211 in NS and 423 in HC, suggesting greater reduction of albumin-bound $25(\text{OH})\text{D}$ in comparison to the free fraction in nephrotic patients.

In the NS group, the total $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}$ levels were higher in males (22.61 ± 9.9 versus 19.10 ± 6.31 nmol/L; $p = 0.029$ and 53.49 ± 47.89 versus 44.44 ± 64.76 pmol/L; $p = 0.045$ respectively). No difference in bioavailable fraction of $25(\text{OH})\text{D}$ and $1,25(\text{OH})_2\text{D}$ was noted between males and females, however.

We performed bioavailable Vit D assessment before and after treatment in 14 cases. The levels increased from 1.45 ± 1.11 nmol/L to 2.88 ± 4.41 .

Vitamin D Binding Protein

The circulating levels of DBP was similar in patients with NS and controls (210.00 ± 137.10 versus 243.00 ± 85.65 $\mu\text{g/ml}$, $p = 0.128$). Urinary DBP/creatinine ratio was significantly higher in patients with NS (79.39 ± 151.8 vs 6.84 ± 24.8 , $p < 0.0001$) compared to controls.

Serum albumin and DBP showed a positive correlation ($r = 0.264$; $p=0.008$). The total 25(OH)D showed a significant association with serum albumin ($r = 0.377$; $p<0.0001$) and DBP ($r = 0.447$; $P<0.0001$). Bioavailable 25(OH)D inversely correlated with DBP ($r=-0.446$; $p<0.0001$ respectively).

Bone mineral density and Vitamin D

Among NS patients, BMD correlated with bioavailable 25(OH)D ($r = 0.358$; $P = 0.0002$), but not with the total 25(OH)D ($r = 0.174$; $P = 0.079$) (Figure 1). Patients in the top quartile of bioavailable vitamin D had 13% higher BMD values than those in the bottom quartile. Total and bioavailable 1,25(OH)₂D did not correlate with BMD ($r = 0.131$; $p= 0.206$ and $r = 0.107$, $p=0.295$ respectively). No association of BMD was noted with iPTH. BMD was lower in females as compared to males with NS (0.70 ± 0.10 versus 0.85 ± 0.15 ; $P<0.0001$).

Vitamin D and markers of mineral metabolism

The values are shown in Table 2. There was a significant difference in serum calcium, phosphate, and iPTH values between groups. Serum calcium levels did not correlate with total 25(OH)D ($r = 0.145$; $P = 0.169$), bioavailable 25(OH)D ($r = -0.033$; $P = 0.752$), total 1,25(OH)₂D ($r = 0.132$; $P = 0.209$), bioavailable 1,25(OH)₂D ($r=0.082$, $p= 0.435$) and iPTH ($r=0.065$, $p=0.540$). Serum phosphate levels did not correlate with total or bioavailable 25(OH)D, but showed an inverse association with total 1,25(OH)₂D ($r = -0.220$; $P = 0.036$). iPTH levels correlated inversely with total 25(OH) D ($r = -0.374$; $p <0.0001$) (Figure 2A), and showed a stronger inverse correlation with bioavailable 25(OH)D ($r = -0.457$; $p <0.0001$) (Figure 2B). iPTH levels also correlated inversely with total 1,25(OH)₂D ($r = -0.250$; $P = 0.01$) and bioavailable 1,25(OH)₂D ($r=-0.253$, $p= 0.009$). On multiple regression analysis, iPTH

levels showed independent correlation with female gender, BMD, bioavailable 25(OH)D and eGFR (Table 4).

DISCUSSION

We measured serum levels of total 25(OH)D, 1,25(OH)₂D, DBP, urinary DBP and serum albumin and determined bioavailable 25(OH)D as well as 1,25(OH)₂D levels in a cohort of adults with newly diagnosed primary sporadic NS. The present study had three novel observations: a. demonstration of significant reduction of bioavailable 25(OH)D and 1,25(OH)₂D in patients with newly diagnosed NS, b. correlation of bioavailable 25(OH)D but not of total 25(OH) D with bone mineral density in patients with NS, and c. correlation of total and bioavailable 25(OH)D and 1,25(OH)₂D with circulating iPTH.

To our knowledge, these findings have not been reported previously in patients with NS, and build over previously reported studies in healthy subjects and patients with CKD ^{11, 13, 17} that bioavailable rather than total 25(OH)D, the current standard, is the better measure of vitamin D status with respect to bone health and mineral metabolism.

Patients with NS lose several plasma proteins in the urine. As expected, we found significantly greater losses of DBP, a 56kD protein, in urine of patients with NS compared to controls. As a result of this loss, the serum DBP levels would be expected to be low. However, despite the low total 25 (OH)D, we found the circulating DBP in NS to be similar to that seen in healthy subjects. The data in the literature on changes in the DBP and total 25(OH)D in NS are equivocal. Some studies have reported lower concentrations of DBP ¹⁸, whereas others ¹⁹ have reported unchanged levels. Similarly, total 25(OH)D levels have been found to be decreased or unchanged in patients with nephrotic syndrome ^{1, 20}. The preserved DBP levels could be explained by compensatory increase in synthesis of DBP in the liver ²¹. The circulating DBP

level is also influenced by variations in gene encoding this protein ¹⁷. It is possible that this variation could explain the inconsistencies in the reported findings.

Our work lends support to the concept of bioavailable fraction which actually is the albumin bound hormone combined with the free fraction. This albumin bound hormone is only loosely attached to albumin and is available for biologic action at cellular level ¹¹. The importance of bioavailable fraction in NS is suggested by the fact that despite a short duration of symptoms, those with lower bioavailable 25(OH)D had higher iPTH and lower BMD, suggesting that bioavailable levels may be more important marker of vitamin D status than the total levels of 25(OH)D and 1,25(OH)₂D in patients with untreated NS. Even more importantly, we found strong correlations of bioavailable 25(OH)D and 1,25(OH)₂D with iPTH.

The free hormone hypothesis is already accepted for other steroid-binding proteins such as sex-hormone binding globulin ¹⁵. A study which tested the effect of albumin binding on testosterone bioavailability in hirsute women receiving spironolactone¹² found that bioavailable testosterone increased proportionately with increments in free and albumin bound testosterone. A major portion of the albumin bound testosterone entered tissues under all conditions; with the bioavailable hormone vastly exceeding the unbound testosterone in the injected samples. Bikle et al. ^{9, 10} reported ability of 25(OH)D and 1,25(OH)₂D to bind with DBP and albumin with an affinity constant of $7 \times 10^8 \text{ M}^{-1}$ and $6 \times 10^5 \text{ M}^{-1}$ and $3.7 \times 10^7 \text{ M}^{-1}$ and $5.4 \times 10^4 \text{ M}^{-1}$ respectively.

DBP sequesters vitamin D, preventing its activity, prolonging serum half-life and providing a circulating store of 25(OH)D to meet transient periods of deficiency, thereby regulating the bioavailability of vitamin D. *In vitro* studies have shown that 25(OH)D induced monocyte-

macrophage antibacterial activity is attenuated by the presence of DBP ²². Similar effects have also been demonstrated in keratinocytes for 1,25(OH)₂D induced responses ²³. The conclusion from these studies is that non-classical target cells for vitamin D are dependent on the free, rather than DBP-bound vitamin D ligands. In conjunction with these findings, our data supports the need to define the vitamin D status not just by the total serum levels of 25(OH)D, but on a measure of the bioavailable levels.

Our study has certain limitations. Potential causal relationships between vitamin D and BMD cannot be made because of the cross-sectional design. Longitudinal studies would also be able to show whether achievement of remission can lead to restoration of bioavailable vitamin D levels. Other caveats include single site DEXA at femoral neck and the fact the Vermeulen equation has not been validated for calculation of bioavailable vitamin D level a non-steady state conditions. However, previous studies have used the same equation to calculate bioavailable 25(OH)D in other non-stable conditions like patients who are critically ill and patients on hemodialysis^{13, 24}. Direct measurement of true free and bioavailable levels could help verify the estimated data and improve upon the equations used.

In conclusion, this is the first study to report decreased levels of bioavailable 25(OH)D in NS, and shows a relationship of bioavailable 25(OH)D with BMD and iPTH. The study suggests that bioavailable fraction is a better marker than total 25(OH)D or 1,25(OH)₂D with respect to bone as well as mineral metabolism in patients of NS, lending support to the free hormone hypothesis. Studies assessing the modifying properties of albumin and DBP on vitamin D metabolism will help us in identifying the patients who will truly benefit from vitamin D supplementation.

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Table and Figure Legends:

Table 1. Demographic characteristics of patients with nephrotic syndrome and control subjects.

Table 2: The detailed clinical and biochemical characteristics of patients with nephrotic syndrome and control subjects.

Table 3. Serum levels of total and bioavailable 25 (OH) D and 1,25 (OH)₂D in subjects with nephrotic syndrome and controls.

Table 4: Multiple regression analysis with iPTH as dependent variable

Figure 1. Representative scatterplots showing correlation of BMD with: **A)** serum levels of total 25(OH)D, and **B)** bioavailable 25(OH)D in nephrotic patients (NS)

Figure 2. Scatter plot showing correlation of iPTH with **A)** total 25(OH) D and **B)** bioavailable 25(OH)D in NS subjects